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Original Article

Deriving rabbit embryonic stem cells by small molecule inhibitors

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Abstract: We previously developed pluripotent rabbit embryonic stem cells (rbES) using a culture system supplemented with basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF), noggin and Y-27632 (referred to as iFLY). In present work, we explored multiple approaches to enhance the chance of deriving domed pluripotent rbES cells by inhibition of MEK, GSK, and PKC signaling pathways. Domed stated rbES were derived in defined medium supplemented with 15% KOSR, 10^3 IU/mL mouse LIF, 10 ng/mL bFGF and three inhibitors to the MEK (PD0325901, 1 μM), GSK3 (CHIR99021, 3 μM) and PKC (Gö6983, 5 μM) (3i). Domed rbES were passaged every 3-4 days till passage 3-4 for the designated experiments. We showed that bFGF and LIF are indispensable for the derivation and maintenance of rbES; whereas the 3i medium containing inhibitors to the MEK (PD0325901), GSK3 (CHIR99021) and PKC (Gö6983) were necessary for deriving domed rbES. Domed rbES possessed naïve ES markers as *Rex1* and *ERAS* in addition to *Oct4*, *Klf4*, *Sox 2* and *c-myc* by RT-PCR. Domed rbES showed positive staining for Rex1, Fgf4, Klf4, Nanog and Oct4 by immunofluorescence chemistry. Further deleting either one factor in 3i medium as CHIR99021, PD0325901, Gö6983 or bFGF resulted in disappearing of domed rbES colonies. The optimal concentrations of 3i contained 0.75 μM PD0325901, 2.25 μM CHIR99021, and 4.5 μM Gö6983. Our work, in combination of different inhibitors for deriving rabbit ES, supports that the network of signal pathways plays an important role in ES self-renew, propagation and maintenance, and sheds light on deriving authentic properties of rbES in an important yet understudied model animal species.

Keywords: Rabbit, embryonic stem cells, signal transduction, small molecules

Introduction

Establishing rabbit genome line competent embryonic stem cells (ES) can be utilized for gene targeting to generate animal models for many human diseases such as cardiovascular diseases, stroke, diabetes, Alzheimer's disease, cystic fibrosis, AIDS and retinal degeneration [1, 2]. Although recently developed gene editing such as TALEN [3] and CRISPR/Cas9 [4] can effectively generate knockout and knock-in gene mutations, it is so far inevitable to rule out the possibility of genome off-target mutation and mosaicism in targeted animals [5]. Recently, two-cell homologous recombination-CRISPR can efficiently generated the large insertions of 700-1400 bp fragments franked with homology arms of 1-5 kb DNA lengths at endogenous loci [6]. It is realistic to develop humanized animal models, which a vast large human genome DNA fragment (such as 100 kb or more) can be introduced into targeted animal genome through homologous recombinant on ES [7].

Embryonic stem cells possess the characteristics of self-renew with the plasticity of differentiation into specialized somatic cell lineages and germ lines [8]. ES are proposed as naïve and primed states of pluripotency which defines

the distinction of embryonic cell un-restriction to cell lineage commitment [9]. The ground state of mouse ES self-renewal is established by inhibiting differentiation-inducing signaling mitogen-activated protein kinase (MEK) and glycogen synthase kinase 3 (GSK) [10]. A network of gene transcription regulatory circuitry is delineated for ES naïve pluripotency [8]. Although pluripotent rabbit embryonic stem cells (rbES) have been established since 1994 [11], there is not a naïve state of rbES proven to be germline competent while most of rbES have shown the characteristics of propagation and self-renewal, differentiation into three germ-layers by *in vitro* induction and teratoma formation [12, 13]. We previously derived rbES lines via iFLY medium by supplementing with the cellular factors for signaling pathways, including LIF (LIF/STAT3 pathway), basic fibroblast growth factor (bFGF, FGF/MEK pathway), noggin (bone morphogenetic protein, BMP pathway), and Y-27632 (Rho-associated protein kinase, ROCK inhibitor) [14]. The iFLY derived rbES has represented a morphology of flat cell colonies and pluripotent markers. It is reported that both mouse and rat ES [15, 16] can be derived with 2i medium containing LIF and inhibitors to GSK (CHIR99021) and MEK (PD0325901) with the domed cell colonies and possesses the germ line transmission, respectively. We found that both LIF and bFGF are essentially required for rbES derivation and maintenance. However, 2i medium containing LIF and inhibitors to the GSK (CHIR99021) and MEK (PD0325901) are not sufficient for derivation of rbES lines [14, 17]. It is reported that inhibition of protein kinase C (PKC) signaling by inhibitor Gö6983 (PKCi) maintains mouse and rat ES self-renew, pluripotency and differentiation [18, 19].

We studied the effects of different cellular factors and small molecule inhibitors of signaling pathways including LIF/STAT3, bFGF/MEK, GSK3 inhibitor (GSK3i, CHIR99021), MEK inhibitor (MEKi, PD0325901), JNK (c-Jun N-terminal kinase) inhibitor (JNKi, SP600125) [19-26], PKC inhibitor (PKCi, Gö6983), p38 inhibitor (p38i, SB203580) [19, 27], BMP inhibitor (BMPi, Noggin) [14, 28] and Forskolin (cAMP activator) [21, 22]. We derived rbES lines with three inhibitors to GSKi, MEKi and PKCi [19-21, 25] in the medium supplemented with bFGF and LIF, in which rbES grew as domed cell colonies as that naïve state of mouse ES. We showed that bFGF and LIF are essential for maintenance of domed rbES cells. The molecular analysis revealed that domed rbES cell lines derived by 3i possessed pluripotent makers similar to blastocysts, but distinct of flat rbES derived by iFLY. The success of deriving domed rbES provides the new approach to derive naïve state of rbES for biomedical research and gene targeting.

Materials and methods

Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

Animal maintenance, hormone superovulation, and blastocyst collection

Animal protocol was approved by the Animal Care and Use Committees of Nanjing Normal University (NSD-2013-30). This study was also performed in accordance with the recommendation in the Guided for the Care and Use of Laboratory Animals of the National Institutes of Health. Sexually mature (6-12-month-old) New Zealand white female rabbits were superovulated scheduled as three days with two 3 mg, two 4 mg, and two 5 mg injections of FSH (Folltropin, Canada), then by one intravascular injection of 150 IU hCG (Chorulon, USA). Hormone treated females were mated with males two times upon hCG injection. *In vivo* blastocysts were flushed on Day 4.5 post mating with D-PBS with 0.1% PVA, and cultured in 2.5% FBS (HyClone, USA) B2 medium (Laboratories CCD, France) at 38.5°C in 5% CO_2 and humidified air prior to embryo seeding.

Deriving domed rbES with small molecule inhibitors

Embryonic stem cells were derived from rabbit and mouse embryos with different medium conditions. Basically, basal ES medium consisted of KnockOut DMEM (Gibco, USA) supplemented with 0.1 mM non-essential amino acids (Sigma), 0.1 mM β-mercaptoethanol (Millipore, USA), 2 mM GlutaMAX (Gibco, USA), 50 U/mL penicillin, and 50 μg/mL streptomycin (HyClone, USA). iFLY consisted of 20% fetal bovine serum (FBS, HyClone, USA), 100 ng/mL Noggin

Table 1. Deriving rabbit domed ES cells with different celluar factors

Factor	Function	2i	2iB	3i	4i	2iF	iFLY
1 μM PD0325901	MEK inhibitor	+	+	+	+	+	-
3 μM CHIR99021	GSK3 beta inhibitor	+	+	+	+	+	-
5 μM Gö6983	PKC inhibitor	-	-	+	-	-	-
10 μM SP600125	JNK inhibitor	-	-	-	+	-	-
10 μM SB203580	p38 inhibitor	-	-	-	+	-	-
10 ng/ml bFGF	Growth factor	-	+	+	+	+	+
10 μM Forskolin	cAMP activator	-	-	-	-	+	-
Embryonic stem cell derivation		No	No	Domed	No	No	Flatted

Footnote: Basal medium except iFLY as described in materials and mrthods was a knockout DMEM with high glucose contained with 15% KSOR, 10^3 IU/mL mouse LIF, 2 mM GlutaMAX, 0.1 mM MEM NEAA amino acids, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 50 μg/mL penicillin-streptomycin. Rabbit *in vivo* blastocysts were cultured in basal medium supplemented with different signal pathway celluar factors and inhibitors. Flatted rbES were derived in iFLY system seen in Methods.

(Stemgent, USA), 10 ng/mL bFGF (Gibco, USA), 10 ng/mL human LIF (Millipore, USA), and 10 μM Y-27632 (Stemgent, USA) in base medium. The medium included different inhibitors (**Table 1**) was arranged as (1) 2i consisted of 15% KOSR (knockout serum replacement, Gibco, USA), 10^3 IU/mL mouse LIF (Millipore, USA), 3 μM CHIR99021 (Stemgent, USA), and 1 μM PD0325901 (Stemgent, USA), this 2i system was also used to derive naïve mouse ES in our laboratory and served as controls for rbES; (2) 2iB consisted of 2i medium added with 10 ng/ml bFGF (Millipore, USA); (3) 3i medium had the same constituents of 2iB medium, and supplemented with 5 μM Gö6983 (Selleck, USA); (4) 4i included 2iB medium, and supplemented with 10 μM SP600125 (Selleck, USA), 10 μM SB203580 (Selleck, USA), but without Gö6983; (5) 2iF consisted of 2iB, and supplemented with 10 μM Forskolin (Selleck, USA). Flatted rbES was derived by iFLY approach in our laboratory [14] and served as controls for domed rbES. Domed mouse ES was derived from 2i medium and served as the controls for rbES [24].

Prior to embryo seeding, the mucin coats and zona pellucida of blastocysts were removed mechanically using glass pipettes. Embryos were seeded on Mitomycin C treated mouse embryonic fibroblast (MEF) feeders. Five to seven days after plating, cell outgrowths were picked and dissociated into small clumps before re-seeding. Passaging was performed by incubating ES-like colonies with 0.25% Trypsin (Gibco, USA) for 3 min followed by plating small cell clumps at a density of 1×10^3 cells/cm^2 onto new feeders. Passaging was performed at 3-4 day intervals. Dissociated rbES were frozen in cryopreservation medium with 90% FBS and 10% DMSO, and then cells were stored in liquid nitrogen. Domed rbES at passage 3-4 were allocated to further experiments. In order to determine whether any factor in 3i medium was not necessary for deriving domed rbES, one of PD0325901, CHIR99021, Gö6983, LIF and bFGF was deleted in 3i medium (**Table 2**).

Immunocytochemical staining for ES-specific markers

Immunostaining was performed with five ES-specific markers Oct4 (diluted 1:1000, ab-27985, Abcam, USA), Nanog (diluted 1:1000, AntiNanog-001, Lannuo Biotechnologies, China), Klf4 (diluted 1:200, AntiKlf4-011, Lannuo Biotechnologies, China), and Fgf4 (diluted 1:500, AntiFGF4-002, Lannuo Biotechnologies, China) and Rex1 (diluted 1:1000, ab28141, Abcam, USA). Briefly, rbES cells were fixed with 4% paraformaldehyde for 10 min and permeated with 0.2% Triton X-100 (Solarbio, China) and 0.1% Tween 20 (Sangon Biotech, China) for at least 10 min. Cells were then incubated in PBS-T (PBS supplemented 0.05% Tween 20) with 2% BSA for 30 min, followed by incubation with corresponding primary antibodies at 37°C for 1 h. Cells were washed with PBS-T for three times, and incubated with secondary antibody conjugated with FITC (chicken anti-goat IgG-FITC, diluted 1:1000, SC-2998, Santa Cruz, USA; goat anti-mouse IgG-FITC, diluted 1:500, SC-2010, Santa Cruz, USA) at 37°C for 2 h. Finally, cells were washed 5 times with PBS-T, and stained with 10 μg/mL 4',6'-diamidino-

Table 2. The effect on different cellular factors on maintaining domed rabbit ES cells

Factor	Function	3i	3i-PD	3i-CHIR	3i-PKCi	3i-bFGF	3i-LIF
10^3 IU/mL LIF	STAT3	+	+	+	+	+	-
1 μM PD0325901	MEK inhibitor	+	-	+	+	+	+
3 μM CHIR99021	GSK3 inhibitor	+	+	-	+	+	+
5 μM Gö6983	PKC inhibitor	+	+	+	-	+	+
10 ng/ml bFGF	Growth factor	+	+	+	+	-	+
Embryonic stem cells		Domed	No	No	No	No	No

Footnote: Basal medium was a knockout DMEM with high glucose contained with 15% KSOR, 2 mM GlutaMAX, 0.1 mM MEM NEAA amino acids, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 50 μg/mL penicillin-streptomycin. Domed rbES cells at passage 3 were cultured in the basal medium supplemented with different signal pathway celluar factors and inhibitors.

Table 3. Primer sequences for RT-PCR analysis

Target gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR Conditions	Size (bp)
Rex1	CGGCATTCCAAGGCGTTC	CCACCCTCCTTTCTCACGAC	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	163
ERAS	GCCAACGTATACGGAGCCTT	TCAGGCAGCTTTTTGCCAAC	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	85
Oct 4	CCTTCGCAGGGGGGCCTA	CATGGGGGAGCCCAGAGCA	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	161
Klf4	GGGCAAGTTCGTGTTGAAGG	TAACACTGATGACCGACGGG	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	74
Sox 2	CCGCTACGACGTAGCGCG	CGAGCCCATGGAGCCGAGC	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	133
c-Myc	GACGCGTCCTTCTCCCCCA	CTCTGGTTACCATGTCACCG	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	112
GAPDH	GGAGCCAAACGGGTCATCATCC	GAGGGGCCATCCACAGTCTTCT	95°C 15 s, Annealing/Extension 60°C 72 s, 35 Cycles	233

2-phenylindole (DAPI) for 10 min. Cells were observed under inverted fluorescence microscope.

RT-PCR of pluripotent markers

RT-PCR was used to detect the expression of *Rex1*, *ERAS*, *Oct4*, *Klf4*, *Sox2*, *C-myc* and *GAPDH* in rbES derived using iFLY and 3i systems and blastocysts as controls. The primer sets from these pluripotent markers in included in **Table 3**. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, USA) from rbES and blastocysts. Isolated RNA was eluted and used for RT-PCR. RT was carried out using 2.5 IU reverse transcriptase and random hexamers with approximately 0.5 μg total RNA at 20 μL. The solution was incubated at 42°C for 15 min. Four μL of RT products were used in subsequent PCR to amplify transcripts of those pluripotent genes for ES. The PCR reaction was carried out in 20 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 mM primers, and 0.4 unit Taq DNA polymerase. PCR was initiated at 95°C for 2 min followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. All PCR reactions including the appropriate internal positive and negative controls were repeated three times. PCR product of 10 μL was analyzed on 1.5% agarose gel.

Karyotyping

The cells were incubated in growth media supplemented with 100 ng/ml colcemid (KaryoMax Colcemid Solution, 15212-012, Invitrogen, USA) for 2-3 h at 38.5°C in 5% CO_2. Cells at passage 5 were then trypsinized and pelleted at 1500 rpm for 5 min, suspended in 6 ml of 75 mM KCl and kept in 37°C water bath for 10 min. The cells were centrifuged and fixed in acetic acid/methanol (v/v=1:3) for 10 min. The centrifugation and fixing steps were repeated three times. For making a slide, the cell pellet was re-suspended in 0.5 ml acid/methanol solution and one drop of cell suspension was dropped onto a pre-chilled clean microscopic slide. The chromosomes were stained with 5% Giemsa (10092013, Invitrogen, USA) for 15 min. The chromosomes were examined at 1000× magnification under oil.

In vitro differentiation of rbES cells into cardiac muscles

The domed rbES at passage 5 were induced to cardiac differentiation. The differentiation

medium consisting of 80% DMEM/F12, 20% FBS, 0.1 mM non-essential amino acids, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol was applied for embryoid body (EB) induction and cardiac differentiation. The rbES colonies were detached from 6-well culture plates by incubating with 1 mg/ml dispase (Gibco, USA) solution at 37°C for 5-10 minutes. The differentiation medium was used to dilute and stop enzyme reaction and the dispase was removed by centrifugation. After re-suspending in the differentiation medium, the colonies were dissociated by slowly pipetting into small cell clumps and then placed in ultra-low attachment plates (Corning, USA) for 5 days of suspension culture. The emerged EBs were picked up and plated on a well of 6-well culture plates coated with 0.1% gelatin. The contracting cells were expected to observe after cultivation with the differentiation medium for additional 7-10 days.

Statistical analyses

The densities of domed rbES colonies in different inhibitor concentrations were determined and those data were transformed by an arc sine transformation. The transformed data then were analyzed by ANOVA (General Linear Model, SPSS 11.0, Chicago, IL). A p value <0.05 was considered significant.

Results

Deriving domed state rbES with CHIR99021, PD0325901 and Gö6983 inhibitor

We developed several rbES lines using an iFLY culture system containing bFGF, LIF, noggin, Y27632 and FBS (**Table 1**). These rbES lines form large, flat colonies (**Figure 1A**) morphologically distinct from domed mouse ES colonies (**Figure 1B**). bFGF is critical for deriving and maintaining these rbES lines, as its withdrawal from culture leads to immediate cell differentiation [14]. We then seeded rabbit blastocysts onto MEF feeders in a 2i system containing CHIR99021 (GSK inhibitor), PD0325901 (MEK inhibitor) and LIF which is effective for deriving and maintaining naïve mES capable of germline transmission (**Figure 1B**). Although rabbit embryonic cells formed domed like structures on day 2 post seeding in 2i, which may indicate the capturing of a putative ground-state cell population, cell death began on day 4 [14].

We next attempted to derive domed rbES in a 3i culture system containing CHIR99021, PD0325901, and Gö6983 (PKC inhibitor) in addition to LIF and bFGF. Using this system, domed rbES (**Figure 1C**, passage 5), were derived from rabbit blastocysts with a morphology similar to that of naïve mES (**Figure 1B**; **Table 1**). Likewise, we tried to derive rbES by combining different inhibitors and cell factors, defined as 2i, 2iB, 3i, 4i and 2iF (**Table 1**), only domed rbES were derived from rabbit blastocysts in 3i medium. RT-PCR revealed that these domed rbES expressed typical pluripotent marker genes *Oct4*, *Sox2*, *Klf4*, *C-myc* similar to rabbit blastocysts and iFLY rbES (**Figure 1D**; **Table 3**, passage 4 for 3i, passage 4 for iFLY and rabbit blastocyst on D4.5). Strikingly, naïve-state pluripotent markers *ERAS* and *Rex1* that are uniquely expressed in naïve mouse ES [9, 21] were highly expressed by domed rbES and rabbit blastocysts but not by iFLY rbES. Further domed rbES at passage 4 were stained positive with Oct4, Nanog, Klf4, Fgf4 and Rex1 antibodies, respectively (**Figure 2**). These results indicated that domed rbES could be derived toward a naïve state. The domed rbES showed 65% normal karyotype with 42+XY chromosomes (**Figure 1E**, n=44). Furthermore, EB formation showed domed rbES had differentiated into cardiac beating cells (**Figure 1F**).

Domed rbES were indispensably dependent upon three small molecule inhibitors

Upon deriving domed rbES with 3i medium, we proposed to derive rbES by deleting one inhibitor/cell factor. As a result, there was not any rbES colonies maintained from any combinations by eliminating one factor of CHIR99021, PD0325901, and Gö6983, LIF and bFGF (**Table 2**). We further cultured domed rbES in modified 3i systems with concentrations of the three inhibitors reduced to 1/8, 1/4, 1/2 and 3/4 of that in the original 3i medium. The original concentrations of 3i medium consisted of 1 μM PD0325901, 3 μM CHIR99021, and 5 μM Gö6983. We observed no domed rbES colonies in basal medium (Base-3i), but gradually increased densities of domed rbES colonies with greater inhibitor concentrations (P<0.05;

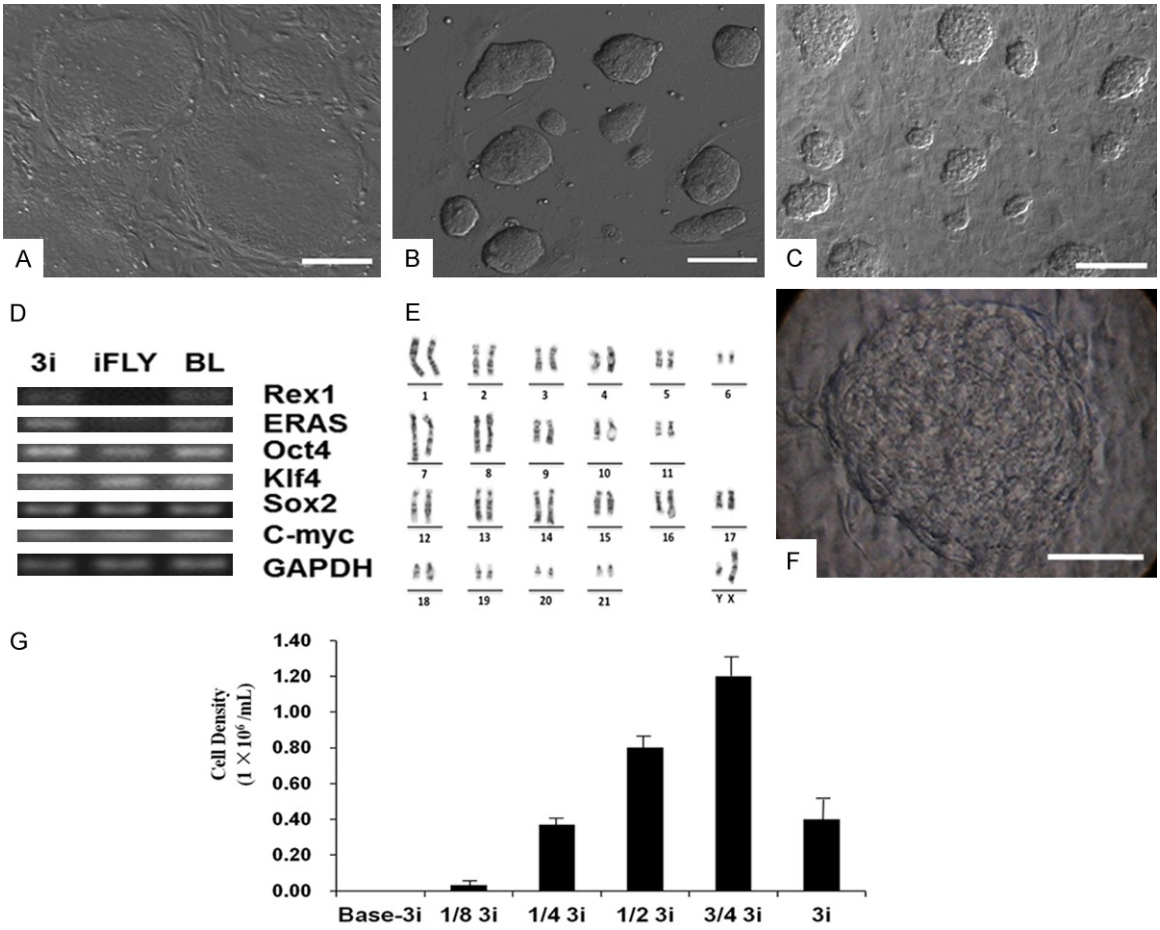

Figure 1. Rabbit ES derived from iFLY and 3i medium. A. Flat colonies of rbES derived from iFLY. B. Naïve state and domed mouse ES derived by 2i (PD0325901, CHIR99021) proven to be germline transmission in our laboratory. C. Domed rbES colonies from 3i (PD0325901, CHIR99021, Gö6983). D. RT-PCR of pluripotent marker genes on domed rbES at passage 4 in 3i, iFLY at passage 4 and rabbit blastocyst on D4.5. Marker genes *Rex1*, *ERAS* RNAs were expressed in domed rbES (3i) and blastocysts while absent in flat rbES (iFLY). *Oct4*, *Klf4*, *Sox2*, *C-myc* were expressed in rbES in both conditions. E. The G-banding karyotype of domed rbES at passage 5, the domed rbES were identified as 42+XY, with 65% normal karyotype (n=44). F. Domed rbES at passage 5 were differentiated into cardiac beating cells after directed cardiac induction. G. The effect of concentration of 3i on growth of domed rbES. Domed rbES at passage 4 were cultured in ES medium supplemented with different concentrations of 3 inhibitors. The original of 3i ES medium contained 1 μM PD0325901, 3 μM CHIR99021 and 5 μM Gö6983. Base-3i was ES basal medium added with LIF + bFGF, but without 3 inhibitors served as controls. Bar=50 μm.

Figure 1G, passage 3). However, domed rbES colony density was similar between the original 3i medium and 1/4 3i culture medium (P>0.05; **Figure 1E**), indicating that higher concentrations of inhibitors may be toxic to rbES. The optimal concentration of inhibitors was 3/4 of the original 3i (**Figure 1G**), which were 0.75 μM PD0325901, 2.25 μM CHIR99021, and 4.5 μM Gö6983.

Discussion

In this study, we have demonstrated that domed rbES can be derived by small molecules inhibiting MEK (PD0325901), GSK (CHIR99021) and PKC (Gö6983) signal pathway. The domed rbES were morphologically distinct from those derived from iFLY (**Figure 1A**), but resemble to mouse naïve ES routinely established in our laboratory (**Figure 1B**). Acquiring the ground or naïve state of rbES has been very challenging. We previously reported the establishment of pluripotent rabbit rbES lines using the FL and iFLY [14] system. These cells, as all other rabbit ES and induced pluripotent stem cells (iPSCs) reported to date share typical properties of primed stem cells and all failed to transmit into germ cells [29, 30]. Although the ground state

of mouse ES is established by only inhibiting MEK and GSK, which proves LIF is dispensable for naïve mouse ES [10], naïve mouse and rat ES characterized by the help on LIF/STAT3 signaling pathway and independency on FGF for maintenance of pluripotency, can be efficiently derived and maintained in 2i/3i-LIF conditions containing LIF and inhibitors to GSK, MEK pathways, readily contribute to the germline cells [31-33]. To date, mouse and rat remain the only mammalian species in which germline transmitting ES are available, despite large amount of work by many groups to derive naïve ESs in a number of non-rodent species.

It is necessary that the collaboration of multiple and different gene regulatory circuity is pivotal for ES self-renew [8], especially for deriving and maintaining domed state rabbit ES in our study. Small molecule inhibitors to multiple signaling pathways, except MEK and GSK, have been explored in non-rodent species [34]. Recent work found that inhibitors to JNK, P38, PKC, ROCK, BMP, BRAF and SRC signal pathway are beneficial for naïve human ES [35, 36]. In the present work we demonstrated that combined treatment of three inhibitors to MEK, GSK, and PKC had significantly promoting effects on the maintenance of domed rbES after different combination of small molecule inhibitors. Either two inhibitors of MEK (PD032590), GSK (CHIR99021) (2i) [15, 16, 37] or two inhibitors plus bFGF (2iB) [14, 17] could not support rbES formation. Likewise, adding 4 inhibitors (4i) as MEK (PD032590, GSK (CHIR99021), JNK (SP600125), P38 (SB203580) together with bFGF [19-23, 25, 26] was not successful for deriving rbES, neither did MEK (PD032590), GSK (CHIR99021) together with Forskolin (2iF) [21, 22] (**Table 1**). It is noticeable that PKC inhibitor (Gö6983) in collaboration with the inhibitors of MEK (PD032590), GSK (CHIR99021) remarkably promoted the formation and growth of domed rbES colonies. This indicates that PKC inhibitor plays a major role in the network of signal pathways. It is reported that inhibition of PKC signaling promotes rat ES self-renewal and contributes germline transmission [19]. PKCi, Gö6983, can inhibit PKCz-NK-κB-microRNA-21/microRNA-29 regulatory axis which maintains rat ES-specific epigenetic modification of pluripotency genes, in turn, results in their gene expression [19]. In addition, the synergistic inhibiting of three signal pathways (MEK, GSK and PKC) is necessary for isolation and maintenance of domed rbES. Our study revealed that further deleting one of any factors (PD032590, CHIR99021, Gö6983 or bFGF) resulted in a failure of rbES derivation. It is noticed the fact that PD032590 inhibits MEK and CHIR99021 inhibits GSK to release β-catenin is necessary for maintaining naïve pluripotent states [21, 38]. In this study, domed rbES need both bFGF and PD032590, it seems that its mechanistic regulation is contradictory, because bFGF stimulates MEK signal pathway while PD032590 inhibits the same pathway. One explanation addresses that the complex process of signal pathways exist in rabbit ES, that is FGF signal pathway have dual functions that stimulating both MEK and AKT (protein kinase B, PKB) pathways [29, 39]. Ying et al. (2008) reported that combination PD184352 and SU5402 inhibitors could significantly decrease phosphor-ERK levels, but FGF activated both PI3K (phosphatidylinositol 3-kinase)/Akt (Protein Kinase B) and Ras-MEK signaling cascades [10]. In other words, although PD032590 inhibits MEK signal pathway that induces primed ES process [21], bFGF functions as an alternative regulation of PI3K-AKT pathway that is important for gene activation [39, 40]. Actually PI3K/Akt signal transduction pathway is a famous mediator of growth promoting and cell survival cascade, it is actually present and functional in mammalian preimplantation embryos [41]. Likewise, it cannot be excluded the possibility that PD032590 may not complete block MEK pathway, and it is reasonable that the partial blockage of MEK may be helpful for naïve related gene expression. In the future study, we will dissect this complicated signaling network. However, our study has clearly demonstrated that combination of different inhibitors in deriving rabbit domed ES which possess the properties of naïve pluripotent markers, and it supports the hypothesis that the network of signal pathways plays an important role in ES self-renew, propagation and maintenance. By reducing the concentration of inhibitors in original 3i medium (1 μM PD0325901, 3 μM CHIR99021, and 5 μM Gö6983) to that of 1/8, 1/4, 1/2 and 3/4 of original concentrations, the domed cell colonies increased significantly from base-3i medium (without 3i) (**Figure 1G**). We further confirmed that the best concentrations of 3i medium for maintaining domed rbES contained the concentrations of inhibitors as

0.75 μM PD0325901, 2.25 μM CHIR99021 and 4.5 μM Gö6983. The original usage of 3i reduced the rbES cell density, indicating the high concentration of inhibitors may be toxic for domed rbES. The further study may be needed to reduce individual inhibitor in the combination to acquire the better deriving efficiency.

It was recently shown that deriving and maintaining naive human and monkey ES require bFGF [35, 36], which for a long time has been considered as a factor for deriving primed state ES, based on knowledge gained from mouse and rats. We found that bFGF and LIF are also indispensable for the derivation as well as the maintenance of rbES. Without bFGF supplementation, no putative rbES were derived in both iFLY and 3i medium. Consistently, rbES with domed shaped morphology were derived and maintained using 3i medium supplemented with bFGF and LIF. Withdrawing of bFGF in the culture medium caused immediate differentiation of the rbES in both domed (3i) and flat (iFLY) rbES colonies [14]. These evidences show similar bFGF requirements in both human and rabbit ES. It has been shown that, comparing to mouse embryos, rabbit embryos have a more similar pattern of preimplantational development and lineage formation to that of human embryos [42]. For example, in human and rabbit embryos, Oct4 expression was detected at 8-cell stage in both human [43] and rabbit embryos [42] indicating both human and rabbit embryos possess zygotic genome activation (ZGA) at same embryonic stage, and it is further present in both the inner cell mass (ICM) and the trophectoderm (TE) cells [42, 44]; whereas in mice Oct4 is expressed at 2-cell stage and restrictively expressed in the ICM, but not in the TE of a blastocyst [45, 46].

The domed rbES were not only morphologically resemble to naïve mouse ES, but also showed with naïve ES markers such as *Rex1* and *ERAS* [34] in addition to those common ES pluripotent makers, *Oct4*, *Klf4*, *sox2* and *c-myc* (**Figure 1D**) [34]. We routinely derive mouse ES with 2i medium (PD032590, CHIR99021) that contributes to germline transmission in our lab (Du et al. unpublished data). The expression pattern of molecular markers was similar between rabbit blastocysts and domed rbES. In contrast, rbES derived from iFLY medium possessed large and flat cell colonies, and naïve *Rex1* and *ERAS* markers were not expressed. Likewise, naïve cell makers available in our laboratory, such as Rex1, Klf4 and Fgf4 [9], were shown in domed rbES (**Figure 2**), but not in flat rbES (data not shown). These significantly different morphological and molecular properties between domed (3i) and flat (iFLY) rbES provide evidences toward a naïve state rabbit ES. We further differentiates domed rbES into cardiac beating cells *in vitro*. Although primed ES can have the similar potential, this capability of differentiation from domed rbES proved the possibility of further differentiation. Further *in vivo* differentiation testing was not yet performed in this study due to funding limitation. In future study, we will perform a large chimera and ES complementation study to examine its *in vivo* differentiation and test the potential of germ line transmission. However, the results and detailed information from this study provides the scientific community a novel approach and inherent mechanism for the purpose of deriving authentic rabbit ES, a very important species to human disease model.

It is believed that Yamanaka factors, which were originally reported in induced pluripotent stem cells, may improve the chance of deriving naïve rbES [47]. Exogenous expression of four factors (Oct4, Klf4, Sox2, c-Myc) or modified combinations of some factors in embryos or cells are capable of inducing somatic cells into naïve state in some species. Overexpression of Yamanaka factors in pig ES enhanced the derivation of naïve-like ES [48]. Likewise, introduction of these factors in the derivation process led to the establishment of naïve-like rabbit iPSCs [49]. This approach may be particularly useful for initial derivation and/or conversion of primed rbES cells to naïve states [26]. We envision that optimized application of three categories of factors (i.e. growth factors, inhibitors and Yamanaka factors) would ultimately lead to the development of authentic rabbit ES: First, use Yamanaka factors to induce or reset the cells into a naïve pluripotent status; second, supply growth factors (e.g. LIF and bFGF) to sustain the self-renewal; and third, use inhibitors of differentiation to prevent the cells exit from the naïve state.

In summary, we have successfully derived domed rbES cells with the properties of naïve ES by three small molecules (3i) inhibiting MEK (PD032590), GSK (CHIR99021) and PKC (Gö6983) signal pathway. Embryonic rbES derived by 3i medium possess the similar morphological and molecular characteristics to mouse

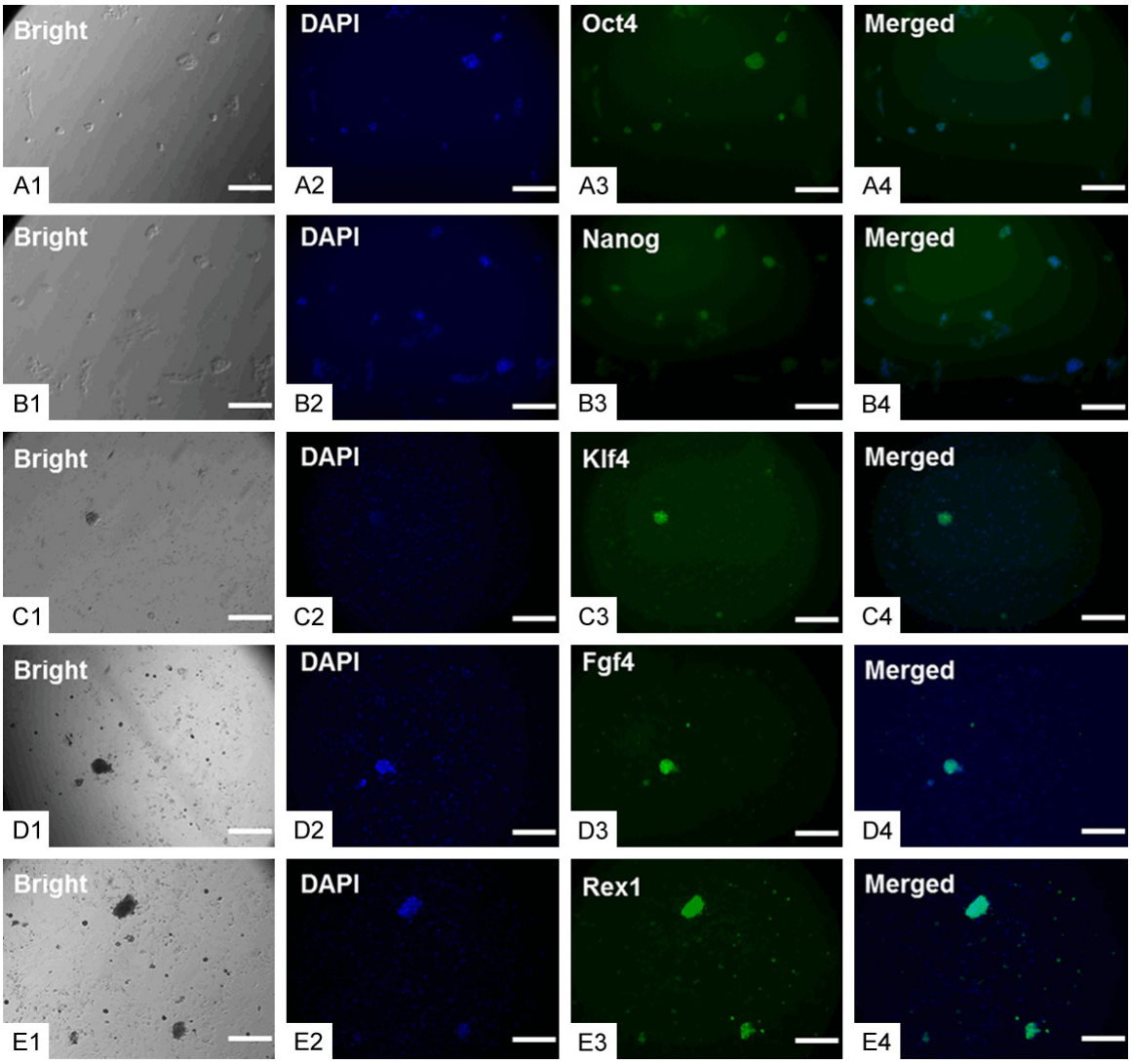

Figure 2. Immunochemistry characterization of domed rbES cells derived and maintained in 3i system. Immunostaining of domed rbES at passage 5 was performed by first antibody to Oct4 (1:1,000,000) (A1-A4), Nanog (1:1000) (B1-B4), Klf4 (1:200) (C1-C4), Fgf4 (1:50) (D1-D4) and Rex1 (1:1,000) (E1-E4), subsequently by secondary antibody Goat IgG H&L (FITC) (1:1000). The rbES showed positive staining of five pluripotent stem cell markers, while Fgf4 and Rex1 were mouse naïve ES markers. Bar=100 μm.

naïve ES, but much distinct to flat rbES derived from iFLY [14]. This may pave the way to derive naïve rbES, which are very important for study of rbES differentiation into germline transmission and finally toward therapeutic and genetic modification as unique models for many human diseases.

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Disclosure of conflict of interest

None.

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